Treatment of *Pseudomonas aeruginosa* Biofilm–Infected Wounds with Clinical Wound Care Strategies: A Quantitative Study Using an In Vivo Rabbit Ear Model

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Background: Bacterial biofilm is recognized as a major detriment to wound healing. The efficacy of traditional wound care against biofilm has never been studied. The authors evaluated the effect of clinical strategies against biofilm-infected wounds in a quantitative, in vivo model.
Methods: Using a rabbit ear biofilm model, wounds were inoculated with *Pseudomonas aeruginosa* or left as uninfected controls. Inoculated wounds acted as untreated controls or underwent treatment: every-other-day sharp débridement (I), lavage (II), Silvadene (III), or lavage and Silvadene (IV), or initial débridement with daily lavage and Silvadene (V). Wounds were harvested on days 12 and 18. Histological wound healing parameters and viable bacterial counts were measured. Biofilm structure was studied with scanning electron microscopy.
Results: Uninfected controls healed better than *P. aeruginosa* biofilm–infected wounds across all parameters ($p = 0.01$). Groups IV and V demonstrated improved healing ($p = 0.05$) and decreased bacterial count ($p = 0.05$) compared with untreated *P. aeruginosa* biofilm, whereas groups I through III showed no differences in either. Scanning electron microscopy following a group V treatment showed temporary disruption of biofilm structure, which reformed in 24 hours.
Conclusions: Pseudomonal biofilm markedly impairs wound healing, shown quantitatively using our in vivo model. Despite common practice, wound care strategies cannot restore biofilm wounds to a healing phenotype when used alone or infrequently. The durability of biofilm extends nonhealing wound chronicity, thus requiring aggressive, multimodal therapy aimed at reducing bacterial burden. The authors' novel, rigorous study validates critical principles applicable to all clinical wound care. (*Plast. Reconstr. Surg.* 129: 262e, 2012.)

Chronic wounds represent a significant and growing problem facing health care professionals today. In 2008, health care costs associated with chronic wound care management were estimated to be upward of $25 billion annually in the United States.[1–6] Given their longstanding nature, the impact on patient lifestyle, financial security, and overall well-being is an immeasurable burden that requires a renewed effort toward innovative solutions.[7,8] Several common disease processes are classically associated with nonhealing wound pathogenesis, including cardiovascular disease, diabetes mellitus, obesity, and peripheral vascular disease,[9–14] each of which involves underlying, fundamental injury pathways such as hypoxia, ischemia-reperfusion injury, and venous stasis.[10,15,16] These abnormalities continue to serve as the focus for ongoing, intense clinical and scientific investigation.

Despite the aforementioned research, a previously unknown but critical component of impaired

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wound healing has recently risen to the forefront: bacterial biofilm.[15,17–22] Defined as a complex community of aggregated bacteria embedded within a self-secreted extracellular polysaccharide matrix, biofilm is the predominant state of bacteria[23] found throughout the body (e.g., gastrointestinal tract, dental enamel) and in association with infected foreign materials (e.g., implanted breast prostheses, urinary tract catheters).[18,24,25] With regard to chronic wounds, bacterial biofilm has been implicated as an independent contributor to delayed wound healing through several in vitro, in vivo, and clinical models.[18,20,26–33] In particular, the emergence of sophisticated imaging and molecular sampling techniques over traditional culture-dependent methods has demonstrated the presence of chronic wound biofilms several times over[18,20] and that the amount of bacteria within these wounds is often underestimated.[20,34]

Distinguishing bacterial biofilm from classically studied free-floating, or "planktonic," bacteria, are its inherent defense and survival mechanisms. These include their enhanced resistance to inflammatory cell phagocytosis[35,36] and antibiotics[24,37,38]; their use of dynamic, protective cell-to-cell communication, termed quorum-sensing[18,39]; and their ability to shed planktonic bacteria to establish new biofilm populations.[18,19] The remarkable durability of biofilm, when considered alongside its newfound role in chronic wound pathogenesis, represents an additional level of complexity associated with treating nonhealing wounds. This growing difficulty stands in stark contrast to present-day chronic wound management, which has shown little evolution, remaining steadfast with traditional methods such as débridement lavage, and antimicrobials as part of wound bed preparation.[40–46] Although practitioners remain fiercely loyal to these well-known treatments, there is little evidence that they improve chronic wound healing in a quantitative manner.[47,48] Furthermore, despite the emerging role of biofilm as a significant mediator of delayed wound healing, there are no studies that address the efficacy of clinical wound care strategies on in vivo biofilm-infected chronic wounds.

In this study, we aim to quantify the effects of clinical wound care management on biofilm-infected wounds using our established, in vivo, rabbit ear, wound biofilm model.[49] Using reproducible quantitative endpoints including histologic healing and viable bacterial count measurements, we compare the efficacy of conventional wound care against established *Pseudomonas aeruginosa* biofilm, a common species within chronic wounds.[22] We hypothesize that interval use of single-modality treatments will result in only minimal improvements in both healing and biofilm burden. However, we propose that combining therapies, and increasing their frequency of use, will result in corresponding improvements in a dose-dependent manner.

MATERIALS AND METHODS

Animals

Under a protocol approved by the Animal Care and Use Committee at Northwestern University, adult New Zealand White rabbits (aged 3 to 6 months and weighing approximately 3 kg) were acclimated to standard housing and fed ad libitum. All animals were housed in individual cages under constant temperature and humidity with a 12-hour light/12-hour dark cycle. A total of 35 rabbits were used during this study.

Bacterial Strains

P. aeruginosa laboratory strain PAO1 was obtained from the laboratory of Dr. Barbara H. Iglewski (University of Rochester Medical Center). *P. aeruginosa* was grown on *Pseudomonas* Isolation Agar (Hardy Diagnostics, Santa Maria, Calif.) overnight at 37°C and co-cultured in Luria broth at 37°C until log-phase was achieved. An optical density at the 600-nm wavelength was measured. Optical density at the 600-nm wavelength of 0.5 was equivalent to 10^6 colony-forming units per microliter as determined preempirically.

Wound Protocol and Bacteria Biofilm Model

The wounding protocol and development of biofilm-infected wounds is based on our previously published model of in vivo wound biofilm in the rabbit ear.[49] In brief, rabbits were anesthetized with intramuscular injection of a ketamine (22.5 mg/kg) and xylazine (3.5 mg/kg) mixture before surgery. Ears were shaved, sterilized with 70% ethanol, and injected intradermally with 1% lidocaine/1:100,000 epinephrine at the planned wound sites. Six, 6-mm-diameter, full-thickness dermal wounds were created on the ventral ear down to the perichondrium and dressed with Tegaderm (3M Health Care, St. Paul, Minn.), a semiocclusive transparent film. Individual wounds were either left sterile as uninfected controls or inoculated with 10^6 colony-forming units of *P. aeruginosa* at postoperative day 3. Bacteria were allowed to proliferate under the Tegaderm dressing. Topical ciprofloxacin 0.3% (Ciloxan; Alcon, Fort Worth, Texas) was applied at postoperative day 4 to eliminate free-floating, planktonic-phase

bacteria, leaving a predominately biofilm-phase phenotype. To prevent seroma formation and regrowth of planktonic bacteria, thus maintaining a biofilm-dominant infection, an antimicrobial, absorbent dressing containing polyhexamethylene biguanide (Telfa AMD; Tyco Healthcare Group, Mansfield, Mass.) was applied to biofilm wounds at postoperative days 5 and 6 and then every other day until harvest. All dressings were checked daily throughout the protocol.

Wound Treatment Plan

Clinical treatments were administered to *P. aeruginosa* biofilm–infected wounds either every other day or daily starting at postoperative day 6, the time at which a steady-state, predominantly biofilm infection is present. After each treatment, new Telfa and Tegaderm dressings were reapplied. Rabbit ears were assigned to one of five treatment types before wounding. Treatments included every-other-day sharp débridement (group I), every-other-day low-pressure water lavage (group II), every-other-day topical Silvadene (Smith & Nephew, London, United Kingdom) (group III), every-other-day low-pressure water lavage followed by topical Silvadene (group IV), and initial wound débridement at postoperative day 6 followed by daily low-pressure water lavage and topical Silvadene (group V). Sharp débridement was completed using a no. 15 scalpel (Becton Dickinson AcuteCare, Franklin Lakes, N.J.), removing any purulent exudate and debris from the wound bed. A Waterpik Cordless Water Flosser (Waterpik, Fort Collins, Col.) was used to simulate low-pressure water lavage, and approximately 0.1 cc of Silvadene Cream 1% (Monarch Pharmaceuticals, Bristol, Tenn.) was applied topically to any wounds in a treatment group requiring Silvadene.

Harvesting of Wounds

Animals were euthanized with intracardiac Euthasol (Virbac AH, Inc., Fort Worth, Texas) injection. Wounds were harvested for several analyses, including histologic analysis using hematoxylin and eosin staining at multiple time points: postoperative day 6 before the first treatment, postoperative day 12, and postoperative day 18. For viable bacterial count measurements, wounds were harvested at postoperative day 12 and postoperative day 18 for drop dilution and counting of live bacteria. In addition, scanning electron microscopy was performed to visualize the wound bed surface. Biofilm wounds before treatment; immediately after initial débridement, lavage, and Silvadene therapy; and 24 hours after treatment were imaged.

Histologic Analysis

With a 10-mm punch, wounds were excised and bisected at their largest diameter for hematoxylin and eosin staining. Tissues were fixed in formalin, embedded in paraffin, cut into 4-μm sections, and stained for analysis under a light microscope. Slides were examined for quantification of epithelial and granulation gaps and total granulation area using a digital analysis system (NIS-Elements Basic Research; Nikon Instech Co., Kanagawa, Japan) as described previously.[50] Two blinded, independent observers evaluated all histologic sections. The results of both examiners were averaged. Slides were omitted if results differed more than 30 percent among examiners.

Viable Bacterial Counts Using the Drop Plate Method

Wounds were harvested for live bacterial counts. The dermal layer on the dorsal ear was removed and wounds were excised with a 10-mm punch. Tissue samples were collected into separate MagNA Lyser Green Beads tubes (Roche Diagnostics, Indianapolis, Ind.), each containing 1 ml of phosphate-buffered saline. All samples were homogenized at 5000 rpm for 90 seconds (MagNA Lyser; Roche Diagnostics) and then sonicated (Microson Ultrasonic Cell Disrupter; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) for 2 minutes at 6 to 8 W to disrupt any biofilm present. The resulting solutions were serially diluted and plated on *P. aeruginosa* Isolation Agar plates and incubated overnight at 37°C. The number of colony-forming units was determined by standard colony counting methods.

Scanning Electron Microscopy

To determine biofilm structure, wound samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.2), washed three times in phosphate-buffered saline, and dehydrated through an ethanol series and hexamethyldisilazane. Samples were mounted by means of double-sided tape to specimen stubs, followed by gold-platinum (50:50) ion coating (108 Auto Sputter Coater; TedPella, Inc., Redding, Calif.). Samples were visualized using a Carl Zeiss (Oberkochen, Germany) EVO-40 scanning electron microscope operated at the scanning voltage of 10 kV.

Statistical Analysis

Data are presented as mean ± SE and were analyzed using the *t* test (two-tailed and unpaired) to compare control, untreated *P. aeruginosa* biofilm–infected wounds, and individual treatment groups. Kruskal-Wallis one-way analysis of variance for nonparametric values was used to compare differences in wound healing and viable bacterial counts between all experimental groups. The level of significance was set at $p < 0.05$.

RESULTS

Using our in vivo biofilm model, an initial comparison was made histologically between uninfected control and untreated *P. aeruginosa* biofilm–infected wounds at postoperative day 12 (Fig. 1). Biofilm-infected wounds demonstrated marked differences in epithelialization and granulation tissue formation as compared with controls, similar to the effects of *Staphylococcus aureus*, demonstrated in previous iterations of the model.[49] These differences were highly significant ($p < 0.001$) when quantified through measurement of epithelial (0.29 mm versus 4.29 mm) and granulation (0.38 mm versus 5.05 mm) gap and new granulation tissue area (332.5 mm^2 versus 49.3 mm^2) (Fig. 2). Biofilm structure was assessed using scanning electron microscopic imaging. Untreated *P. aeruginosa* wounds at postoperative day 12 demonstrated individual bacterial cells embedded within a well-developed extracellular polysaccharide matrix that covered the wound bed surface (Fig. 3).

To assess the efficacy of clinical wound care strategies, biofilm-infected wounds were subjected to different conventional treatments, designated as groups I through V as described previously. Histologically, wounds exposed to combinations of treatment modalities (groups IV and V) and/or increased frequency of treatment (group V) demonstrated the greatest improvements in wound healing (Fig. 4, *fourth row* and *below*). In contrast, wounds treated with single-modality, low-frequency treatments (groups I through III) showed only minimal differences in healing as compared with untreated biofilm-infected wounds (Fig. 4, *above* and *second* and *third rows*). Quantifying these differences, wounds in groups IV and V had significant ($p < 0.05$) improvements in all measured histologic parameters compared with the untreated group (epithelial gap, 3.46 mm and 2.89 mm versus 4.29 mm; granulation gap, 3.95 mm and 3.35 mm versus 0.38 mm; and new granulation tissue area, 109.0 mm^2 and 155.9 mm^2 versus 49.3 mm^2, respectively) (Fig. 5).

Viable bacterial count measurements were performed to correlate the improvements seen in group IV and V wounds with decreased biofilm burden. At postoperative day 12, there was no significant difference in bacterial burden between untreated biofilm-infected wounds and wounds in treatment groups I through III (Fig. 6). Meanwhile, groups IV and V experienced the greatest reduction in viable bacteria, both compared with untreated wounds ($p < 0.05$) and overall among all treatment groups. Furthermore, the significant reduction in biofilm burden among these wounds

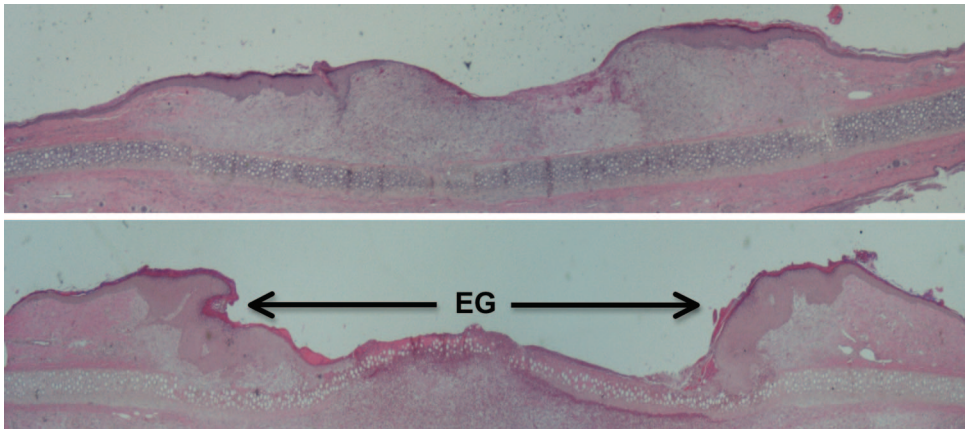

Fig. 1. Histologic comparison of uninfected, control wounds (*above*) and untreated *P. aeruginosa* biofilm–infected wounds (*below*). The image obtained at postoperative day 12 of infected wounds shows distinct impairments in epithelial and granulation gap and new granulation tissue as compared with uninfected controls (hematoxylin and eosin; original magnification, × 20). *EG*, epithelial gap.

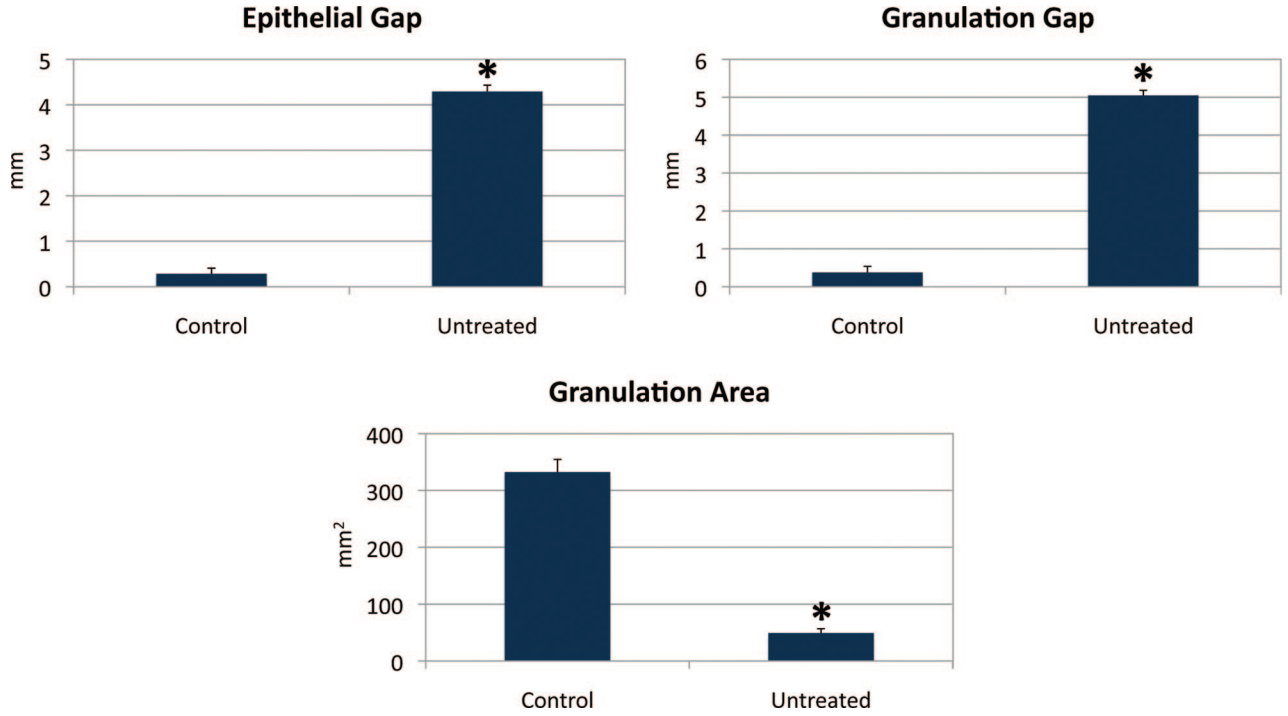

Fig. 2. Quantification of wound healing parameters for uninfected, control wounds and untreated *P. aeruginosa* biofilm–infected wounds. Biofilm-infected wounds demonstrate significant impairment across all measured parameters, including epithelial gap (*above*, *left*), granulation gap (*above*, *right*), and new granulation tissue area (*below*) as compared with control wounds, validating the role of biofilm in delayed wound healing (*$p < 0.001$) ($n = 18$ to 20 wounds per group).

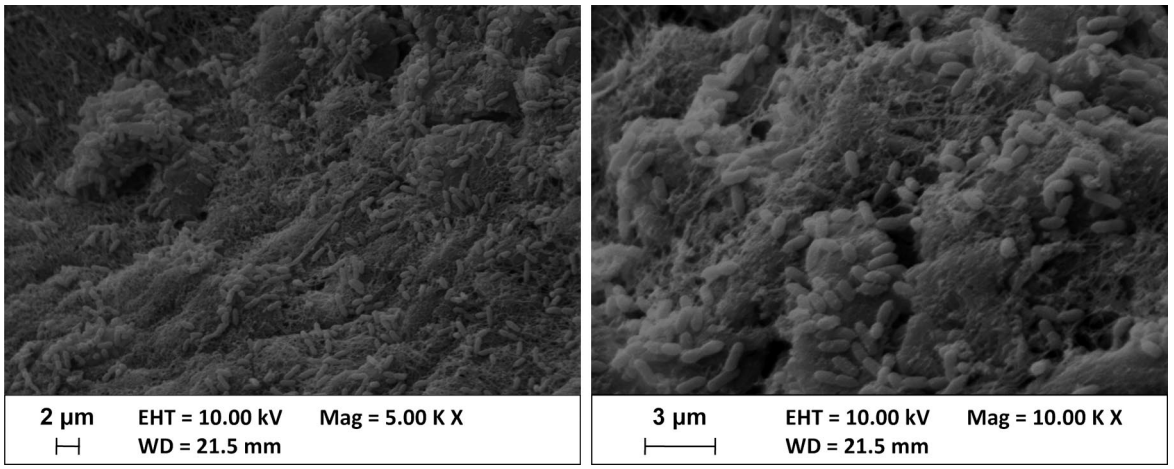

Fig. 3. Scanning electron microscopic images of *P. aeruginosa* biofilm–infected wounds at low (*left*) and high (*right*) magnification, demonstrating the presence of *P. aeruginosa* biofilm within infected wounds at postoperative day 12. Note the numerous individual rod-shaped bacteria encased by a lattice-appearing matrix, completely covering the previously bare wound bed cartilage.

directly corresponded to the improvements seen in total wound healing. One-way analysis of variance of all experimental groups revealed that the trends seen in each wound healing parameter and bacterial counts were all statistically significant ($p < 0.0001$ and $p = 0.0011$, respectively) (Table 1). In addition, analysis of variance postanalysis comparisons demonstrated that group IV and V treatments were the only statistically superior treatments (i.e., there were no differences between any of the single-modality treatments in either wound healing or ability to decrease bacterial burden).

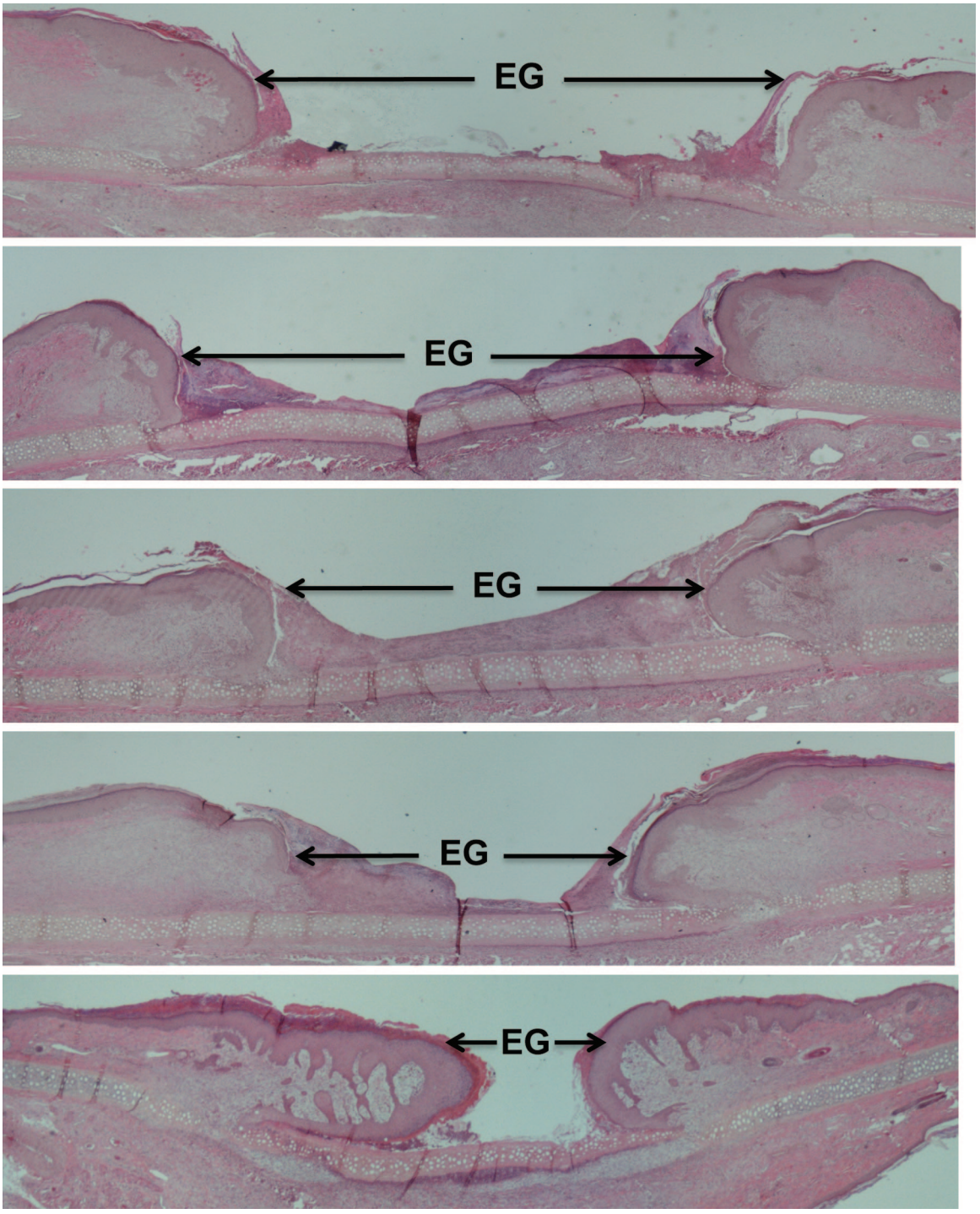

Fig. 4. Histologic comparison of *P. aeruginosa* biofilm–infected wounds following different conventional treatments. Images of wounds at postoperative day 12 following one of five predesignated treatment groups: every-other-day sharp débridement (group I) (*above*), every-other-day water lavage (group II) (*second row*), every-other-day topical Silvadene (group III) (*third row*), every-other-day water lavage plus Silvadene (group IV) (*fourth row*), and initial débridement followed by daily water lavage plus Silvadene (group V) (*below*). Treatments were started when a steady-state biofilm was established at postoperative day 6. Note the increased amount of epithelialization and granulation tissue in group IV and V wounds as compared with groups I through III (hematoxylin and eosin; original magnification, ×20). *EG*, epithelial gap.

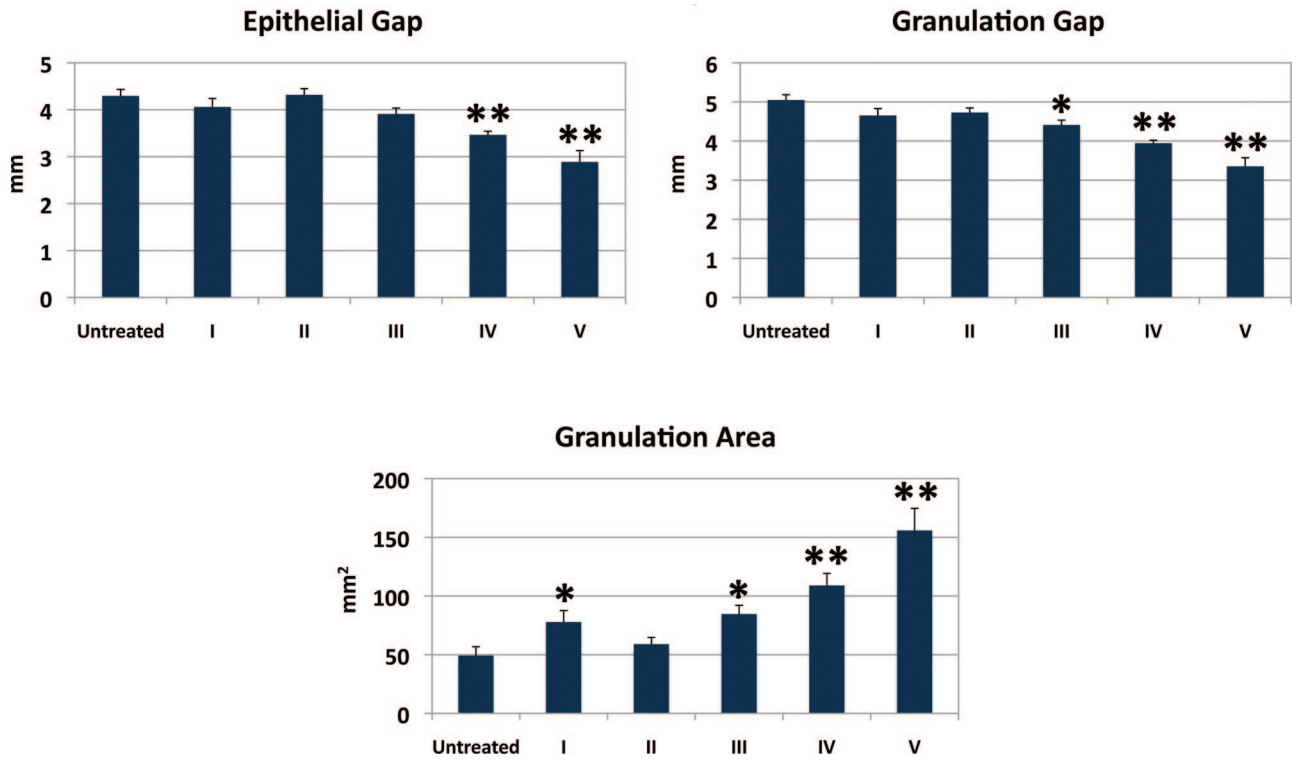

Fig. 5. Quantification of histologic parameters for treatment group wounds as compared with untreated, *P. aeruginosa* biofilm–infected wounds. Multimodality (groups IV and V) and increased frequency group (group V) wounds demonstrated significant improvements in epithelial gap (*above*, *left*), granulation gap (*above*, *right*), and new granulation tissue area (*below*) compared with untreated biofilm-infected wounds (*$p < 0.05$; **$p < 0.001$) ($n = 10$ to 20 wounds per group).

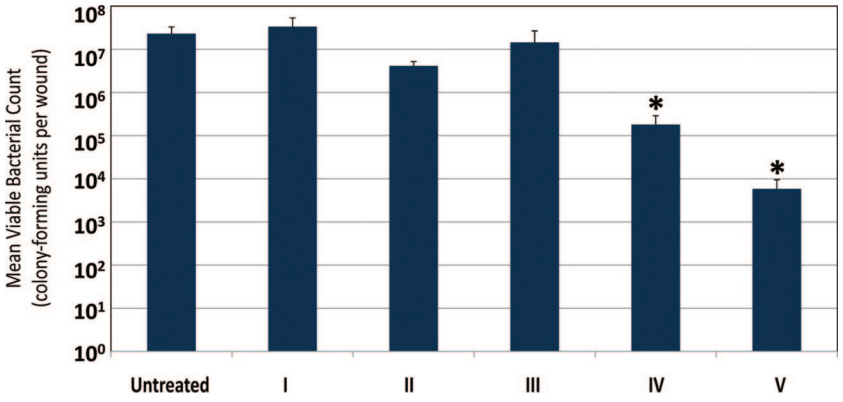

Fig. 6. Bar chart showing mean viable bacterial counts following different conventional treatment groups as compared with untreated, *P. aeruginosa* biofilm–infected wounds. Wounds in groups I through III, each an intermittent, single-modality treatment, demonstrated no differences in bacterial burden at postoperative day 12 as compared with untreated, *P. aeruginosa* biofilm–infected wounds. Groups IV and V had significant decreases in biofilm burden, indicating that increased-frequency combination therapies are effective at reducing bacterial burden (*$p < 0.05$) ($n = 8$ to 10 wounds per group).

Differences in wound healing and viable bacterial counts at postoperative day 12 were validated with additional experiments followed to a longer time point, postoperative day 18 (Figs. 7 and 8). Given the similarities seen at postoperative day 12 between single-modality treatments (groups I, II, and III), comparisons were performed only among untreated biofilm wounds, group II lavage

Table 1. Statistical Comparison of Treatment Groups with One-Way Analysis of Variance

Group Comparison					
Group 1	Group 2	Epithelial Gap	Granulation Gap	Total Granulation Area	Viable Bacterial Counts
Untreated	I	NS	NS	NS	NS
Untreated	II	NS	NS	NS	NS
Untreated	III	NS	NS	NS	NS
Untreated	IV	*	†	‡	‡
Untreated	V	†	†	†	‡
I	II	NS	NS	NS	NS
I	III	NS	NS	NS	NS
I	IV	NS	‡	NS	‡
I	V	*	†	*	‡
II	III	NS	NS	NS	NS
II	IV	*	‡	*	NS
II	V	†	†	†	NS
III	IV	NS	NS	NS	NS
III	V	‡	‡	‡	NS
IV	V	NS	NS	NS	NS
Overall p		<0.0001	<0.0001	<0.0001	0.0011

NS, not significant.
*$p < 0.01$.
†$p < 0.0001$.
‡$p < 0.05$.

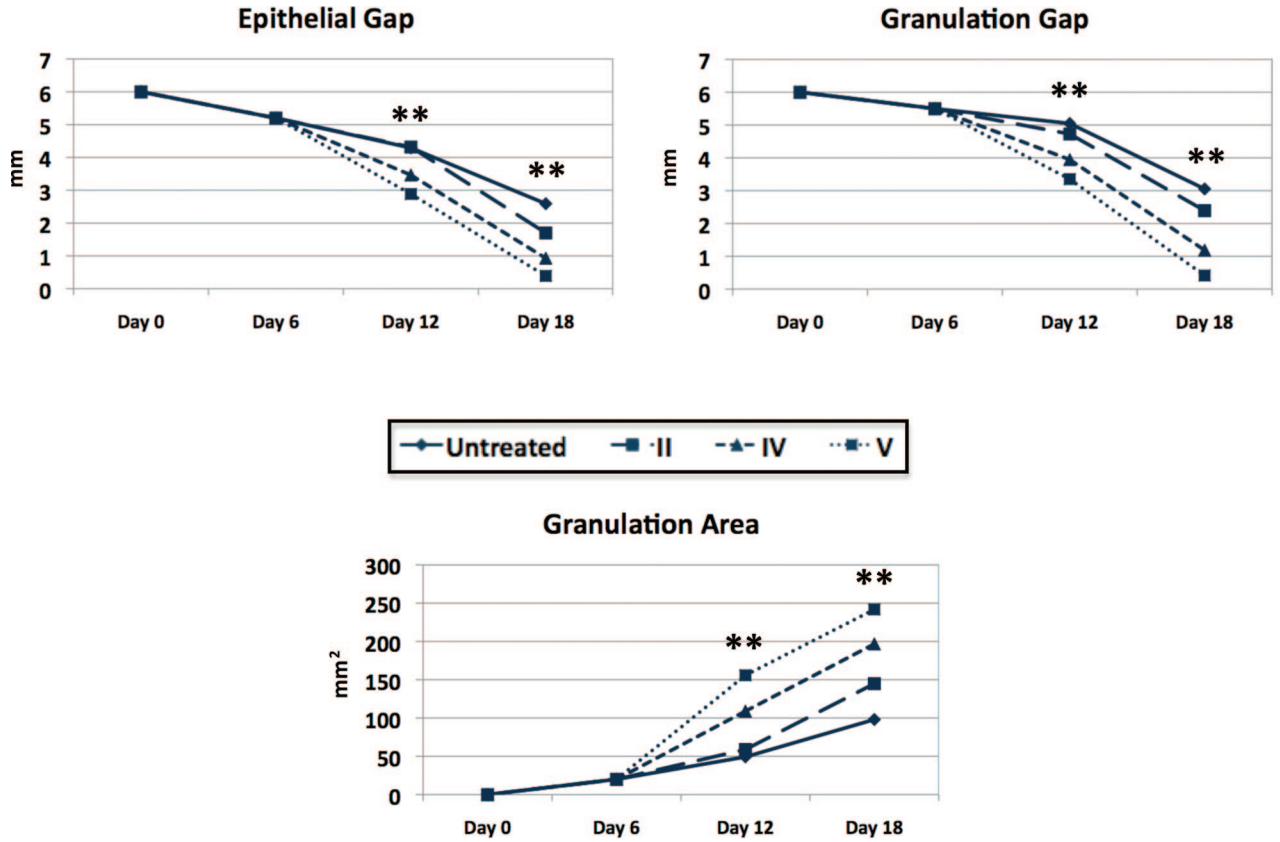

Fig. 7. Long-term quantification of histologic wound healing parameters between untreated, *P. aeruginosa* biofilm–infected wounds and treatment group wounds. Wounds followed to postoperative day 18 maintained trends similar to postoperative day 12, including significant differences between untreated wounds and all treatment groups in epithelial gap, granulation gap, and granulation area. As at postoperative day 12, group IV and V wounds demonstrated the greatest improvements in all healing parameters at postoperative day 18 (**$p < 0.001$) ($n = 10$ to 20 wounds per group).

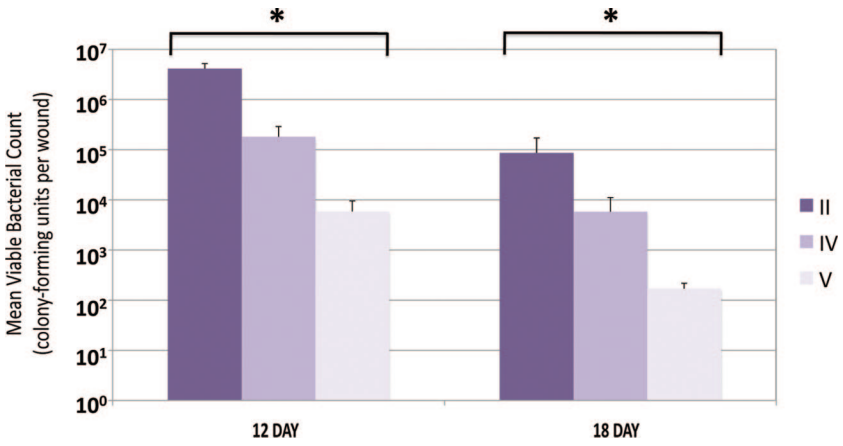

Fig. 8. Histogram showing long-term comparison of mean viable bacterial counts in single-treatment and multimodality treatment groups. All treatment groups demonstrated decreases in bacterial counts over time from postoperative day 12 to postoperative day 18. Similar to postoperative day 12, multimodality treatment groups IV and V demonstrated the greatest overall decrease in viable wound bacteria at postoperative day 18 while maintaining a statistically significant difference between all three groups on analysis of variance (*$p < 0.05$) ($n = 8$ to 10 wounds per group).

wounds, and group IV and V multimodality treatment wounds. As seen at postoperative day 12, one-way analysis of variance demonstrated statistically significant differences among all four groups at postoperative day 18 in terms of epithelial gap, granulation gap, and granulation area ($p < 0.001$) (Fig. 7). In particular, the trends seen at postoperative day 12 were maintained at postoperative day 18, in that multimodality treatment (group IV and V) resulted in greater improvements in all healing parameters, with the increased frequency of treatments in group V having the greatest effect. Similarly, viable bacterial counts also maintained statistically significant differences among the three treatment groups at postoperative day 18 ($p < 0.05$) (Fig. 8). Bacterial counts decreased from postoperative day 12 to postoperative day 18 in all three groups, with groups IV and V maintaining the largest overall decrease in bacterial viability.

Analysis of biofilm morphology both before and after the most effective treatment, found to be an initial débridement followed by daily lavage and topical Silvadene, was performed using scanning electron microscopic imaging. Wounds before treatment revealed distinct *P. aeruginosa* cells within an organized matrix (Fig. 9, *above*, *left*). Immediately following this treatment combination, this well-developed structure appeared disrupted (Fig. 9, *above*, *right*), with minimal evidence of surviving bacteria. However, 24 hours after treatment, the biofilm was reestablished within the wound bed, including an increased presence of extracellular polysaccharide matrix (Fig. 9, *below*).

DISCUSSION

The management of chronic wounds continues to be a burdensome and difficult problem, complicated by both intrinsic host morbidity and environmental exposures.[1–16] Critical to their pathogenesis, bacterial biofilm is increasingly being recognized as a pervasive and robust component of the tissue destruction and delays in healing that are classically seen in chronic wounds.[18,20,26–33] Although chronic wounds are by definition difficult to treat, the presence of biofilm further impairs the efficacy of clinical wound care strategies through its inherent resistance mechanisms.[18,24,34–39] Nevertheless, methods such as débridement, lavage, and topical antimicrobials remain the standard for clinical wound care but are unproven in their ability to accelerate chronic wound healing and reduce bacterial biofilm burden. Using our in vivo wound biofilm model,[49] we wanted to determine whether these standard, conventional wound care strategies are effective against biofilm through a quantitative and rigorous analysis.

This study further validates the ability of our model to create and maintain bacterial biofilm, using *P. aeruginosa* instead of *Staphylococcus aureus* to demonstrate significant differences in wound healing between uninfected control and biofilm-

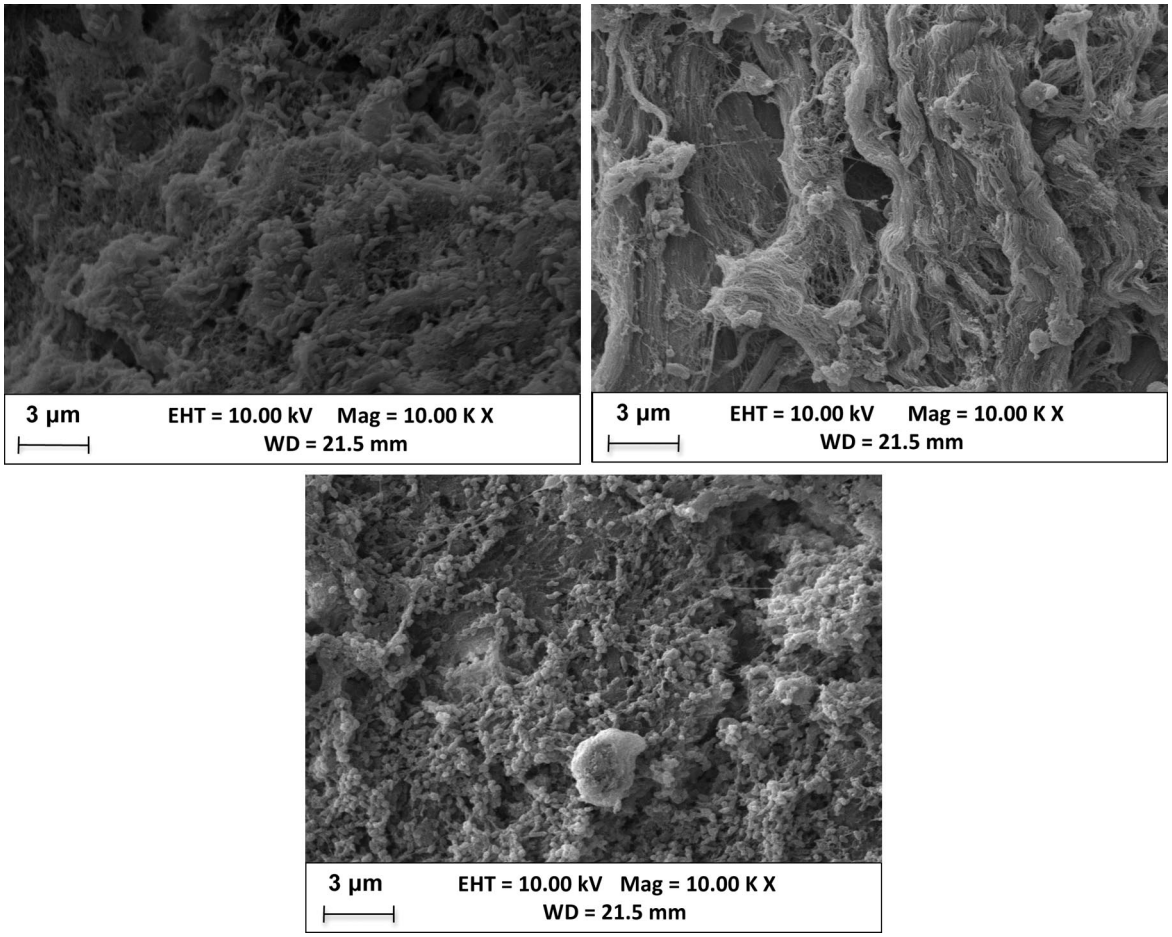

Fig. 9. Scanning electron microscopic images obtained before, immediately after, and 24 hours after treatment of *P. aeruginosa* biofilm–infected wounds with débridement, lavage, and topical Silvadene (group V treatment). Established biofilm before treatment (*above*, *left*) is disrupted using the most effective treatment studied, group V, leaving a wound bed with minimal evidence of bacteria (*above*, *right*). Harvesting of wounds 24 hours after the same treatment demonstrates the return of well-developed biofilm matrix components encasing numerous, rod-shaped *P. aeruginosa* (*below*).

infected wounds. Furthermore, we verify biofilm structure and viability through scanning electron microscopic imaging and measurement of bacterial counts, respectively. When exposed to a variety of traditional wound care regimens, consistent improvements in healing and level of bacterial burden were seen only following multimodality and/or increased-frequency treatment strategies. These differences were statistically significant when compared with untreated biofilm-infected wounds and when weighed against all experimental groups studied at multiple time points. Nevertheless, scanning electron microscopic imaging also reveals that even the most superior wound care strategy cannot completely eradicate biofilm with only one iteration, demonstrating the durability of biofilm and indicating the need for persistent and aggressive therapy.

Previous studies of clinical wound care have demonstrated only mixed efficacy, relying on anecdotal and clinical evidence rather than rigorous investigation. Wolcott and Dowd report on a patient with a dorsal foot burn who underwent topical Nanogel (Teknimed, Hautes Pyrenees, France) Acticoat (Smith & Nephew, London, United Kingdom) treatment combined with oral antibiotic treatment to achieve significant healing after 4 weeks.[51] Meanwhile, Percival et al. demonstrated that silver-containing dressings decreased total bacterial burden when used against an in vitro biofilm[52]; however, no experiments were performed in vivo or with an endpoint of wound healing. Others have looked at different, topical antimicrobial bandages, which showed only mixed efficacy in reducing *S. aureus* burden in partial-thickness porcine wounds.[53] Payne et al. studied enzymatic débriding agents in infected,

granulating rodent wounds, demonstrating rapid decreases in viable bacteria and accelerated healing rates,[47] but did not test these compounds against established wound biofilm. Overall, to date, there remains no definitive consensus on the efficacy of different clinical treatments, particularly with regard to wound healing and bacterial burden. Furthermore, despite the role of bacterial biofilm in chronic wound pathogenesis, there are no studies that have investigated the efficacy of these treatments against an established in vivo biofilm.

In contrast to current literature, our study represents the first investigation of traditional wound care against in vivo bacterial biofilm, including measures of wound healing, bacterial burden, and visualization of biofilm morphology. Surgical dogma has always dictated aggressive and repetitive treatment as part of chronic wound care, although this has never been proven beyond anecdotal evidence and clinical experience.[42–47,51–54] We demonstrate that only through multimodality treatments and increased treatment frequency can quantifiable improvements be seen in the healing of biofilm-infected wounds, thus validating classic surgical teachings. Furthermore, the lack of differences between any single-modality treatment groups indicates that no individual treatment is superior to another, emphasizing the need to combine therapies to achieve positive results.

Underlying these improvements in healing is the ability of intense clinical therapy to disrupt and destroy bacterial biofilm, consistent with the growing consensus that biofilm is critical to delaying keratinocyte migration and wound granulation.[18,20,26–33] We have demonstrated that a single round of multimodality treatment can disrupt biofilm architecture immediately following therapy but that this effect is short-lived, as bacteria reestablish an extracellular polysaccharide matrix within 24 hours. Furthermore, although group V wounds showed significantly more healing than uninfected, control wounds, there was only a 2-log difference in bacterial burden. Although reducing biofilm has a positive effect on would healing rates, these results also reinforce the need for therapy to be directed and persistent to achieve continued improvement.

Unfortunately, the reality in hospitals and long-term care facilities is that many patients do not receive the dedicated care required for their chronic wounds. One can infer from our results that without adequate therapy, an infected chronic wound will not demonstrate meaningful improvements in healing, with the level of biofilm burden remaining unchanged. Extrapolating further, it is conceivable that in an acutely infected wound, such as an infected postoperative incision, there is the potential for developing a more chronic-phase wound without aggressive, multimodal therapy. This may be particularly true in the impaired host, where an ineffective inflammatory response can allow a predominantly planktonic-phase, active infection to persist and transition into a biofilm-phase, chronic infection. This is further enhanced by inadequate, intermittent wound care, which only temporarily disrupts the infectious process, forcing bacteria into a defensive and resistant biofilm state as an evolutionary, protective mechanism. Such a situation is analogous to bacterial drug resistance from improper or incomplete antibiotic use. In both cases, eradicating the infection becomes more difficult.

Despite the rigorous approach, there are limitations to our study. Although our model recapitulates biofilm-infected wound conditions,[49] in future experiments we plan to thoroughly investigate these therapies in an impaired host (e.g., ischemia or ischemia-reperfusion), which can be accomplished in the rabbit ear model.[54] In addition, we also limited our study to a single bacterial species, *P. aeruginosa*. For future studies, polymicrobial biofilm infections will be used to investigate the durability of one type of biofilm over another. Furthermore, we are limited in our ability to perform multiple treatments per day or follow long-term regimens, given the need for rabbit sedation before each procedure. In contrast, the majority of these methods can be performed at the bedside in humans with minimal periprocedural pain, allowing for flexibility in treatment plans. We believe that our results, with statistically significant trends despite only a limited number of treatment iterations in an animal model, indicate the need for aggressive and multimodal therapy to achieve success in treating nonhealing wounds.

Moving forward, it is important to recognize the commitment required to perform effective clinical wound care. As our understanding of biofilm pathophysiology improves, therapeutic interventions may be developed that target specific components of the biofilm development and maturation process. One might hypothesize that their efficacy may be limited by the robust biofilm extracellular polysaccharide matrix that renders antibiotics ineffective. However, combining aggressive clinical care, which can temporarily disrupt biofilm structure, with a targeted therapeutic compound that can prevent its reformation, might enhance the overall improvements seen in a synergistic manner. This type of multidimensional

wound care is an area of future research that can be effectively modeled, systematically investigated, and rigorously tested with our in vivo wound biofilm model, thus serving as a translational gateway into future human clinical trials.

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